IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re PATENT APPLICATION of

BRANSTROM et al.

Appln. No. 08/711,961 Group Art Unit: 1805

Filed: September 6, 1996 Examiner: J. Railey

Title: BACTERIAL DELIVERY SYSTEM

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DECLARATION UNDER 37 C.F.R. § 1.132

Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

I, Donata R. Sizemore, a citizen of the United States of America, hereby declare and state as follows:

1. I hold a Ph.D. in Biomedical Sciences from Wright State University and have worked in the fields of molecular biology, immunology, microbiology and vaccinology. I am a member of the American Society for Microbiology and currently hold the position of Project Manager at MEGAN Health, Inc., St. Louis, Missouri.

2. I am an Inventor on Patent Application No. 08/711,961, and am familiar with the prosecution history of the application, including the Office Action issued October 10, 1997.

3. In the patent application, a means for mutating bacteria by deletion of the *asd* gene within the DAP pathway is described. This mutation results in a strain of attenuated bacteria which is unable to synthesize components required for its cell wall. The consequence of this is that such bacteria are able to enter

target eukaryotic cells, but once inside the cells, the bacteria lyse and die, thereby delivering plasmid DNA to the cells.

4. In the application it is disclosed that *Shigella* as well as other species and strains of bacteria would function according to the invention.

5. In the Office Action of October 10, 1997, claims 28-33 and 44 were rejected by the Examiner as not being enabled. It was the Examiner's position that the disclosure was enabling only for claims limited to *Shigella* strains that have been genetically attenuated by inactivation of the wild-type *asd* gene.

6. Testing the possibility of DNA delivery by *Salmonella* had been previously hampered due to the inability of *Salmonella* strains to efficiently maintian high-copy plasmids with pUC origins of replicaton, like that used to discover *Shigella's* ability to deliver DNA. This phenomenon of plasmid instability has been noted by other investigators. Oyston et al. (Infection and Immunity 63: 563-568, 1995) noted that only 0.52 to 0.88% of *Salmonella* colonizing the spleens of infected mice continued to carry a pUC18 plasmid expressing a *Y. pestis* gene. Further data on the instability of pUC-based plasmids can be seen in Table 1. Commercial plasmid pCMVβ, which was used to demonstrate that *Shigella* could be used to deliver DNA was readily lost from *Salmonella typhimurium* strain MGN0238, a strain containing a deletion in the gene encoding *asd*. Growth of this strain in the

presence of ampicillin, which should place pressure on the strain to maintain the plasmid, did not increase the number of plasmid containing colonies, as a decrease in colony forming units per ml (CFU/ml) of 1 to 1.5 logs after only two hours of growth was detected.

7. In order to test the ability of *Salmonella* to deliver expressible DNA in accordance with the present invention the following experiment was performed.

8. A pBR-based (medium-copy of 50-100 copies per bacterium) eukaryotic expression plasmid was constructed. pBR-based plasmids are known to be well maintained in *Salmonella*. Construction of the plasmid was accomplished by cloning the fragment of pCMBβ (illustrated in attached Figure 1) required for expression of β-gal within eukaryotic cells (EcoRI-0bp to HindIII-4531bp) into commercial plasmid pBR322 that had been digested with EcoRI and HindIII. Ligation of these two fragments resulted in plasmid pMEG290, shown in attached Figure 2. Plasmid pMEG290 was well maintained in *Salmonella typhimurium* strain MGN023s (designated MGN1052s) even without the selective pressure of ampicillin (Table 2).

9. Delivery of plasmid pMEG290 to cultured cells was assessed by comparing the level of delivery between *asd* gene deleted strains of *Salmonella* (MGN1052s) and *Shigella* (15GpMEG290). Delivery experiments were conducted as previously

outlined in Example 2 of the present patent application with the exception that bacterial suspensions of MGN1052s were added to the culture medium of seeded wells, not in direct contact with the cells as is required for *Shigella* (non-motile).

10. To more efficiently assess delivery, cells were fixed in situ by a brief treatment, followed by washing with Hank's Balanced Salt Solution (HBSS), and then overlayed with a solution composed of: 1 mg/ml of 5-bromo-4-chloro 3-indolyl-β-D-galactopyranoside (X-gal), 50 mM sodium phosphate, pH 7.3, 20 μM potassium ferricyanide, 20 μM potassium ferrocyanide, 15 μM sodium chloride, and 1.3 mM magnesium chloride. Cell staining of a deep blue was observed by inverted phase microscopy.

11. The number of visually stained cells from two experiments is shown in attached Tables 3 and 4. The level of DNA delivery by the *Shigella* strain varied from 2 to 8 times greater than that of the *Salmonella* strain.

12. The level of invasiveness of the ΔasdA *Salmonella* strain was much greater than that of the ΔasdA *Shigella*, as shown in attached Tables 5 and 6.

13. The presence of DAP in the bacterial suspension during the adherence and invasion step was not required of the *Salmonella* strain, a characteristic which makes MGN1052s a very desirable delivery vehicle. A similar loss of viability at the

24 hour assay time was seen for both the ΔasdA *Salmonella* and *Shigella* strains.

14. These results demonstrate that *Salmonella* is a suitable organism for use according to the invention, and that persons of ordinary skill would be able to construct suitable mutants for use according to the invention.

15. As disclosed in the subject specification on pages 6, 15 and 35, adherence and invasion genes encoded on the large virulence plasmid or chromosomally within *Shigella* can be transferred to other bacterial strains for the purpose of using these strains with their newly acquired abilities to adhere/invade and escape the vacuole as delivery vehicles of DNA. The transfer of these genes to E. coli is demonstrated in the attached references as detailed below.

16. Formal et al. (Infection and Immunity 1:279-287, 1970) first published data on the genetic transfer of *Shigella* antigens to an E. coli K-12 strain.

17. Transfer of the large virulence plasmid from *Shigella flexneri* 2a to an avirulent E. coli K-12 strain has been shown to endow the strain with the ability to invade HeLa cells (Sansonetti et al., Infection and Immunity 39:1392-1402, 1983).

18. Similar results are shown and expanded upon in Formal et al., Infection and Immunity 46:465-469, 1984 and Newland et al., Vaccine 10: 766-776, 1992).

19. These results demonstrate that *E. coli* is a suitable organism for use according to the invention, and persons of ordinary skill in the art should be able to construct suitable mutants for use according to the invention.

20. As disclosed in the subject specification on page 35, *Listeria* should also be a suitable delivery vehicle according to the invention. *Listeria*, like *Shigella*, can adhere to and invade cells, and escape the confines of the vacuole to grow within the cell cytoplasm, as evidenced in the publications summarized below.

21. The attached publication of Portnoy and Jones (Ann. N.Y. Acad. Sci. 730:15-25, 1994) demonstrates that *Listeria* is able to escape the confines of the vacuole, in accordance with requirements of the present invention.

22. *Listeria* also shares the ability of *Shigella* to spread from cell to cell, utilizing the host cell's own actin, as evidenced in the attached publication of Kadurugamuwa et al. (Infection and Immunity 59: 3463-3471, 1991).

23. These results demonstrate that *Listeria* is a suitable organism for use according to the invention, and persons of ordinary skill in the art should be able to construct suitable mutants for use according to the invention.

24. The results described above in *Salmonella*, *E. coli* and *Listeria* demonstrate that persons of ordinary skill in the art

will be able to construct a variety of bacterial mutants which can be utilized in the invention using routine experimentation, and that the invention is not limited to *Shigella*. As stated in the subject specification, the abilities to adhere/invade and escape the vacuole are the desired characteristics of such bacteria, and it will be clear to persons of skill in the art how to select and construct suitable mutants using routine experimentation.

25. In the Office Action of October 10, 1997, the Examiner indicated that the invention is only enabled for mammalian cells.

26. The attached publications demonstrate that the delivery system of the invention would be expected to function in other animal cells.

27. Lawlor et al. (Infection and Immunity 55:594-599, 1987) used a chicken embryo model to assess the virulence of mutants of *Shigella flexneri* defective in iron transport. In this chicken embryo model, *Shigella* grows in the allantoic fluid before invasion of the embryo. Invasive bacteria can then be isolated from the livers of the embryos.

28. Vasselon et al. (Infection and Immunity 59:1723-1732, 1991) utilized primary fibroblast cells from 13-day old chicken embryos to demonstrate the intracellular movement of *Shigella* along highly organized cytoskeletal structures.

29. The work of Lawlor et al. and Vasselon et al. demonstrate that *Shigella* can adhere to and invade avian cultured cells as well as cells within avian hosts.

30. The invasive capabilities of *Salmonella* in both avian and mammalian hosts is demonstrated in the attached references:

Barrow et al., Infection and Immunity 55:388-392, 1987

Barrow et al., Infection and Immunity 62:4602-4610, 1994

Hassan and Curtiss, Infection and Immunity 62:2027-2036, 1994

Keller et al., Infection and Immunity 63:2443-2449, 1995

31. Thus, the methods and compositions of the invention would be expected to be effective in animal cells, and to require no more than routine experimentation.

32. I declare further that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code and such willful false statements may jeopardize the validity of the instant patent specification or any patent issuing thereon.

By Donata L. Sizemore Date 1-9-98